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Adiponectin protects against Toll-like receptor 4-mediated cardiac inflammation and injury

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Aims Adiponectin (APN) is an immunomodulatory and cardioprotective adipocytokine. Toll-like receptor (TLR) 4 mediates autoimmune reactions that cause myocarditis resulting in inflammation-induced cardiac injury. Here, we investigated whether APN inhibits inflammation and injury in autoimmune myocarditis by interfering with TLR4 signalling.

Methods and results APN overexpression in murine experimental autoimmune myocarditis (EAM) down-regulated cardiac expression of TLR4 and its downstream targets tumour necrosis factor (TNF)α, interleukin (IL)-6, IL-12, CC chemokine ligand (CCL)2, and intercellular adhesion molecule (ICAM)-1 resulting in reduced infiltration with cluster of differentiation (CD)3+, CD14+, and CD45+ immune cells as well as diminished myocardial apoptosis. Expression of TLR4 signalling pathway components was unchanged in hearts and spleens of APN-knockout (APN-KO) mice. *In vitro* APN had no effect on TLR4 expression in cardiac and immune cells but induced dissociation of APN receptors from the activated TLR4/CD14 signalling complex. APN inhibited the expression of a TLR4-mediated inflammatory phenotype induced by exogenous and endogenous TLR4 ligands as assessed by attenuated nuclear factor (NF)-κB activation and reduced expression of TNFα, IL-6, CCL2, and ICAM-1. Accordingly, following TLR4 ligation, splenocytes from APN-KO mice showed enhanced expression of TNFα, IL-6, IL-12, CCL2, and ICAM-1, whereas dendritic cells (DCs) from APN-KO mice demonstrated increased activation and T-cell priming capacity. Moreover, APN diminished TLR4-mediated splenocyte migration towards cardiac cells as well as cardiomyocyte apoptosis after co-cultivation with splenocytes. Mechanistically, APN inhibited TLR4 signalling through cyclooxygenase (COX)-2, protein kinase A (PKA), and meiosis-specific serine/threonine kinase (MEK)1.

Conclusion Our observations indicate that APN protects against inflammation and injury in autoimmune myocarditis by diminishing TLR4 signalling thereby attenuating inflammatory activation and interaction of cardiac and immune cells.

Keywords TLR4 • Adiponectin • Autoimmune myocarditis

1. Introduction

Inflammation can generate severe and irreversible damage to the myocardium, because persistent inflammation underlies the pathogenesis and progression of many common cardiovascular diseases such as myocardial infarction (MI), atherosclerosis, hypertrophy, myocarditis, dilated cardiomyopathy, and heart failure.[1]

Toll-like receptors (TLRs) are key recognition components of the innate immune system and also crucial for the activation of adaptive immunity. In addition to their pivotal role in host immune defences against invading pathogens, TLRs are also capable of modulating inflammation following non-infectious stress insults such as ischaemia in various tissues including the heart.[2]

TLR4 specifically recognizes Gram-negative bacterial lipopolysaccharide (LPS). Moreover, it binds to a heterogeneous group of endogenous ligands such as heat shock proteins (e.g. HSP60) and extracellular matrix breakdown products that are typically released into the myocardium in the context of cardiac injury.[2] Binding of such endogenous

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ligands by TLR4 following cardiac injury triggers immune responses and contributes to the origination or augmentation of inflammation.[3] Thus, the TLR4 signalling pathway is an important mediator of autoimmune reactions that cause inflammation-induced injury in the myocardium. In this regard, TLR4 signalling has been shown to mediate myocardial ischaemia/reperfusion (I/R) injury,[4] maladaptive remodelling,[3] increased infarct size and reduced survival after MI.[5] Moreover, TLR4 has been demonstrated to be essential for the induction of experimental autoimmune myocarditis (EAM) by cardiac myosin.[6] Taken together, the TLR4 signalling pathway is considered to be a major contributor for the progression of important inflammation related cardiovascular diseases representing a promising target for therapeutic interventions.

Adiponectin (APN) is an abundant plasma cytokine, that is primarily expressed in adipocytes.[7] Moreover, APN expression has also been shown for endothelial cells, skeletal myocytes, and cardiac cells.[7] APN exists as full-length protein in several oligomeric forms or as proteolytic cleavage fragment in high concentrations of 3–30 μg/mL in human plasma.[7] APN functions as important modulator of immune reactions exerting a wide spectrum of anti-inflammatory effects.[7] Results of *in vitro* experiments demonstrate that APN inhibits the activation of the pro-inflammatory transcription factor nuclear factor (NF)-κB and expression of tumour necrosis factor (TNF)α in endothelial cells.[8] Moreover, APN suppresses immune cell activation,[9] adhesion to target cells,[8] and controls antigen-specific expansion of T cells.[10] Accordingly, results from *in vivo* studies show that APN mediates cardioprotection by modulation of inflammatory responses. APN conferred resistance against cardiac injury following ischaemia[11] and attenuated myocardial damage in viral myocarditis.[12] Moreover, recently it has been shown that APN favours positive outcome in patients with inflammatory cardiomyopathy by inhibiting cardiac inflammation and remodelling.[13]

Here, we investigated whether APN is protective against TLR4-mediated myocardial inflammation and injury. Therefore we examined the effects of systemic APN overexpression on inflammation and apoptosis in the TLR4-dependent animal model of EAM. The underlying mechanisms of APN effects were studied in cardiac and immune cell culture.

2. Methods

2.1 Animals and mouse model of EAM

The mouse model of EAM has been described previously.[14] Replication defective adenoviral vectors (3×10^8 plaque forming units) expressing mouse APN (Ad-APN) or control vectors (Ad-RR5) were injected intravenously into 8–10-week-old female BALB/c mice (purchased from Jackson Laboratories) 1 week before induction of EAM (Day −7) leading to robust transduction and APN expression in the liver that can be detected in the plasma as long as 28 days. For EAM induction, mice were immunized with 200 μL of a 1:1 emulsion of PBS with 1 mg/mL of a heart muscle specific α-myosin heavy chain-derived peptide (MyHC-$\alpha_{614-629}$ [Ac-SLKLMATLFSTYASAD-OH]) in complete Freund's adjuvant (CFA) at Days 0 and 7. Control mice received PBS/CFA only. The EAM animals were randomly allocated to two groups and were intravenously injected with either Ad-APN or control vector (Ad-RR5). Investigators were unaware of the treatment groups of animals. All mice were sacrificed for further examinations at the inflammatory peak of EAM on Day 21 post-immunization.

For cell isolation, APN-knockout (APN-KO) mice and the corresponding C57BL/6 wild-type (WT) mice were purchased from Jackson Laboratories. Neonatal Wistar Harlan rats were purchased from FEM Berlin (Germany). The investigation conformed to the US NIH *Guide for the Care and Use of Laboratory Animals* (8th Edition, published 2011) and was approved by the respective authorities in Basel (Switzerland) and Berlin (Germany)—No: T 0086/10. Before injections and euthanization, animals were anaesthetized by inhalation of 2.0 vol% isoflurane for 5 min using an automatic delivery system. Adequate anaesthesia was tested by monitoring withdrawal response to foot pinch. Mice were euthanized by cervical dislocation and neonatal rats by decapitation.

2.2 RT^{2}Profiler PCR-array and quantitative real-time polymerase chain reaction

RNA from tissues and cultured cells was extracted by using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). RNA integrity was checked by 2100 Bioanalyzer (Agilent Technologies). The RT^2 Profiler mouse chemokines and chemokine receptors PCR-Array System (SABiosciences) was used as suggested by the manufacturer. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by using High capacity cDNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, and TaqMan gene expression assays from Applied Biosystems.

2.3 Cell culture and reagents

Neonatal cardiomyocytes and fibroblasts were prepared from hearts of 1–3-day-old Wistar Harlan rats as described.[15] Neonatal rat and mouse splenocytes were isolated from spleens of 1–3-day-old Wistar Harlan rats or 6–8-week-old male APN-KO and WT mice, respectively. Mouse DCs were enriched by cultivation of isolated bone marrow cells from 6–8-week-old male APN-KO and WT mice as described.[16] Peripheral blood mononuclear cells were isolated from heparinized blood of healthy human donors using ficoll density gradient centrifugation. Recombinant human full-length APN produced in a mammalian expression system was purchased from R&D Systems; determined endotoxin contamination (Kinetic-QCL, Lonza) was <10 pg/μg protein. TLR4-grade LPS was purchased from Enzo Life Sciences, rat fibrinogen from Sigma-Aldrich; endotoxin contamination was <50 pg/mg. BAY 11-7085, NS-398, Rp-cAMP, PD 098 059, and Wortmannin were purchased from Sigma-Aldrich. For all cell-culture experiments, investigators were blinded to the performed treatment of cells.

2.4 Fluorescence-activated cell sorting

Phenotypic analysis of immune cells was performed with fluorescence-conjugated antibodies against murine CD3, CD11c, and CD86 (BioLegend) and human CD3, CD14, CD19, BDCA-1, TLR4, and TNFα (BD Pharmingen). For the measurement of DC induced T cell proliferation 2×10^4 DCs from APN-KO or WT mice and 2×10^5 CFDA (Vybrant® CFDA SE Cell Tracer Kit, Molecular Probes, Invitrogen) labelled allogenic splenocytes from 6–8 weeks old BALB/c mice were co-cultivated for 4 days.

2.5 Protein analysis

NF-κB activation in whole cell lysates was measured using the TransAM NF-κB p65 subunit DNA-binding ELISA (Active Motif)[13] that specifically detects nuclear (i.e. activated) NF-κB. IκBα phosphorylation was determined by immunoblot using anti-Phospho-IκBα (Cell Signaling Technologies) and anti-α-Tubulin (Calbiochem) antibodies. TNFα and CCL2 levels (R&D Systems) in cell-culture media as well as Troponin I levels (Life Diagnostics) in sera were quantified using ELISA kits as suggested by the manufacturers. Cardiac protein expression of CCL2 was measured using the RayBio® Mouse Cytokine Antibody Array 3 (Ray Biotech). Cardiac protein expression of TLR4, ICAM-1, and GAPDH was quantified by immunoblot using anti-TLR4 (Imgenex), anti-ICAM-1 (Santa Cruz), and anti-GAPDH (Cell Signaling Technologies) antibodies. Receptor interactions between TLR4, CD14, and APN receptors were analysed via immunoprecipitation using anti-TLR4 (Imgenex), anti-CD14 (Santa Cruz), and anti-APN-R1 (Phoenix Peptides) antibodies.

2.6 Migration assay

3.0×10^4 cardiomyocytes or fibroblasts were seeded into the lower receiver plate of HTS Transwell® 96-well permeable support systems (8 μm membrane pore size, Corning Life Science), cultured in DMEM containing 0.2% FBS and stimulated with LPS in the presence or absence of APN. For the measurement of immune cell migration towards the cardiac cells, 1.5×10^5 CFDA (Vybrant® CFDA SE Cell Tracer Kit, Molecular Probes, Invitrogen) labelled rat splenocytes were added to the upper insert plate wells. After co-cultivation of cardiac cells and splenocytes for 24 h, cells in the lower receiver plate wells were harvested and resuspended in 100 μL PBS + 2% Flebogamma. Fifteen microlitres of a 1:10 dilution of polystyrene beads (CompBead, BD Biosciences) was added to the samples for normalization. Quantification of migrated splenocytes was performed by fluorescence-activated cell sorting (FACS) analysis gating on the bead population and measuring an uptake of exactly 1×10^4 beads per approach. Counted was the number of CFDA+ cells that were collected in parallel after exclusion of the bead population.

2.7 Analysis of apoptosis

Cardiomyocyte apotosis after co-cultivation with splenocytes (cell number ratio 1:4) was quantified by TUNEL using the In Situ Cell Death Detection Kit TMR red (Roche Applied Science). Co-cultivated splenocytes were removed by washing with PBS. Apoptosis in myocardial cryosections (5 μm) was determined by TUNEL staining using the In Situ Cell Death Detection Kit Fluorescein (Roche Applied Science).

2.8 Statistical analysis

SPSS 20 or SAS 9.3 were used for statistical data analysis. Differential impact of APN depending on stimulation has been modelled via factorial ANOVA with the interaction between treatment and stimulation. The non-parametric ANOVA type analyses by Brunner have been performed in case of normality assumption being violated or Levene's test of homogeneity being significant. Pair-wise comparisons between individual groups were done using the Mann–Whitney *U* test. Differences were considered statistically significant at a two-sided value of $P < 0.05$. No Bonferroni adjustment has been performed.

For sample size calculation, a type I error of 0.05 and type II error of 0.1 were considered acceptable for an animal study. Reduction of the number of CD45 cells in the heart was chosen as the primary endpoint in our study and based on a previous pilot study, we assumed a large effect size through APN. Sample size calculation was performed with the G*Power 3 (University of Düsseldorf, Germany) and resulted in $n = 6$ per group.

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3. Results

3.1 APN overexpression in EAM reduces cardiac expression of TLR4 and its major downstream targets

To investigate the effect of systemic APN overexpression in TLR4-dependent EAM, mRNA and protein expression analysis in the hearts of mice was performed (*Figure 1A* and *B*, Supplementary material online, *Figure S1*). Induction of EAM led to an increase of cardiac expression of TLR4 and several important chemokines (i.e. CC chemokine ligand (CCL)2), pro-inflammatory cytokines (i.e. interleukin (IL)-6, IL-12, and TNFα), and adhesion molecules (i.e. intercellular adhesion molecule (ICAM)-1) that are collectively involved in the induction and progression of EAM. Following APN gene transfer, however, cardiac expression of TLR4 was significantly reduced ($P = 0.016$, EAM RR5 vs. EAM APN, respectively). This reduction was associated with significantly decreased expression of pro-inflammatory cytokines TNFα, IL-6, and IL-12, as well as the chemokine CCL2 and ICAM-1, all representing downstream targets of TLR4 (*Figure 1A* and *B*, Supplementary material online, *Figure S1*). In order to investigate whether APN directly regulates the expression of central components of the TLR4 signalling pathway WT and APN-KO mice were examined. Neither TLR4, myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-ß (TRIF) nor interferon regulatory factor (IRF)3 were differentially expressed in the hearts and spleens of APN-KO mice (Supplementary material online, *Figure S2A* and *B*). Thus, our results indicate that APN attenuates inflammation in EAM by inhibiting TLR4 signalling.

3.2 APN overexpression in EAM attenuates immune cell infiltration and myocardial injury

Chemokines, inflammatory cytokines, and adhesion molecules participate in homing, accumulation, and activation of immune cells in inflamed tissues.[1] Following EAM induction, up-regulation of CCL2 and ICAM-1 expression was observed associated with accumulation of leucocytes within the heart (*Figure 1B* and *C*). Accordingly, down-regulation of CCL2 and ICAM-1 following APN gene transfer was accompanied by reduced cardiac mononuclear cell infiltration. Specifically, cluster of differentiation (CD)3+ T cell ($P = 0.030$) and CD45+ leucocyte accumulation ($P = 0.030$) were diminished in APN overexpressing animals (*Figure 1C*). Furthermore, expression of CD14, a marker for monocytes playing an important role in the progression of EAM,[17] was significantly down-regulated following APN gene transfer (*Figure 1C*). Persistent accumulation and activation of mononuclear cells within the heart is associated with increased tissue injury mediated by cytokines, reactive oxygen species (ROS), and proteolytic enzymes.[1] Therefore, apoptotic cell death in cardiac tissue sections of EAM mice as well as cardiac specific serum Troponin I were assessed. The amount of apoptosis assessed by TUNEL ($P = 0.029$) staining and Troponin I ($P = 0.008$) concentrations were significantly increased in mice following EAM induction (*Figure 2A* and *B*). APN gene transfer, however, significantly attenuated apoptosis ($P = 0.029$) and Troponin I ($P = 0.030$) increase in this model. Taken together, our data indicate that APN inhibits immune cell infiltration, inflammation, and tissue injury in autoimmune myocarditis.

3.3 APN inhibits TLR4-mediated expression of an inflammatory phenotype on cardiac cells

Incubation of cardiomyocytes and fibroblasts as well as immune cells (i.e. CD14+, CD19+, and DCs) with APN had no effect on TLR4 mRNA (data not shown) or protein expression *in vitro* (Supplementary material online, *Figure S2C*) supporting our data in APN-KO mice. Therefore, we investigated whether APN inhibited intracellular TLR4 signal transduction. The chemokine CCL2 and the adhesion molecule ICAM-1 are essential factors for the targeted activation of immune cells during inflammation. Whereas CCL2 plays a major role as a chemoattractant for the infiltration of immune cells into the myocardium, ICAM-1 enables their firm adhesion to cardiac cells.[1] As shown in *Figure 3A*, incubation of cardiomyocytes with LPS, a potent exogenous TLR4 signalling activator, triggered a significant up-regulation of CCL2 and ICAM-1 expression. Similar results were obtained when cells were stimulated by fibrinogen, an endogenous TLR4 ligand that is released in the context of tissue injury (Supplementary material online, *Figure S3A*).

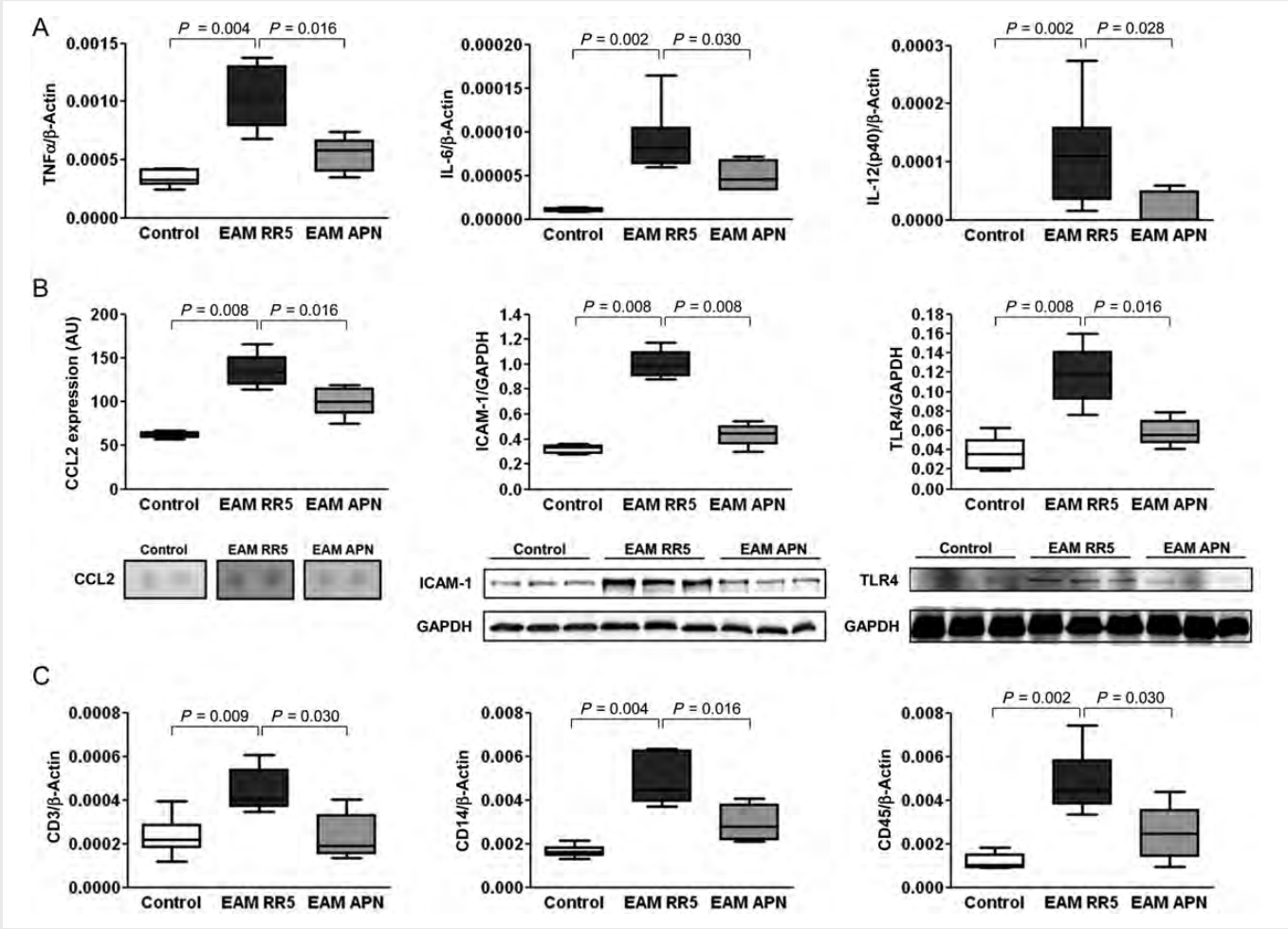

Figure 1 APN overexpression attenuates cardiac inflammation in EAM. Cardiac expression of TLR4 and TLR4 target genes was analysed in mice transduced with a mouse APN expression vector (EAM APN) or control vector (EAM RR5) 21 days after EAM induction. (*A*) mRNA expression of TNFα, IL-6, and IL-12 are shown. (*B*) Representative blots indicating protein expression of TLR4, CCL2 (antibody array), and ICAM-1 are depicted. Box plots indicating quantitative levels. (*C*) mRNA expression of CD3, CD14, and CD45 are shown ($n = 5–6$ animals per group).

However, APN incubation significantly attenuated TLR4-mediated up-regulation of CCL2 and ICAM-1 mRNA expression caused by both LPS and fibrinogen. Moreover, up-regulation of the pro-inflammatory cytokines TNFα and IL-6 after TLR4 ligation by LPS and fibrinogen in cardiomyocytes and fibroblasts was significantly diminished following APN incubation (*Figure 3A* and *D*; Supplementary material online, *Figures S3* and *S4*). Taken together, APN inhibited the expression of a TLR4-mediated pro-inflammatory phenotype on cardiac cells, while APN had no effect in unstimulated cells. These results indicate that APN exerts its anti-inflammatory effects by inhibiting TLR4 signal transduction rather than inhibiting expression of TLR4 or downstream components of its signalling pathway.

3.4 APN inhibits TLR4-mediated NF-κB activation in cardiomyocytes

NF-κB represents the central downstream transcription factor in the TLR4 signalling pathway controlling the expression of major pro-inflammatory targets such as TNFα, IL-6, ICAM-1, and CCL2 (Supplementary material online, *Figure S5*). Following TLR4 ligation by LPS, NF-κB is rapidly activated in cardiomyocytes (*Figure 3B*). APN treatment led to a significant inhibition of TLR4-mediated NF-κB activation ($P = 0.032$) while no APN effect was observed in unstimulated cells. In order to further elucidate the mechanisms involved in the inhibition of NF-κB activation by APN, TLR4-mediated phosphorylation of inhibitor of NF-κB (IκB)α was studied. IκBα phosphorylation primes the molecule for proteosomal degradation leading to nuclear translocation and increased transcriptional activity of NF-κB.[18] TLR4 ligation by LPS caused a significant increase in IκBα phosphorylation. However, APN incubation significantly attenuated TLR4-mediated IκBα phosphorylation (*Figure 3C*). The observed inhibitory effect of APN on TLR4-induced NF-κB activation supports the contention that APN effectively attenuates the TLR4 signal transduction process.

3.5 APN inhibits TLR4 signalling in cardiomyocytes through COX-2-, PKA-, and MEK1-dependent mechanisms

APN-induced effects are among others mediated through phosphoinositide 3-kinase (PI3K),[19] cyclooxygenase (COX)-2, protein kinase A (PKA), and meiosis-specific serine/threonine kinase (MEK)1.[7,8,11] In order to examine their potential role for the inhibitory effects of

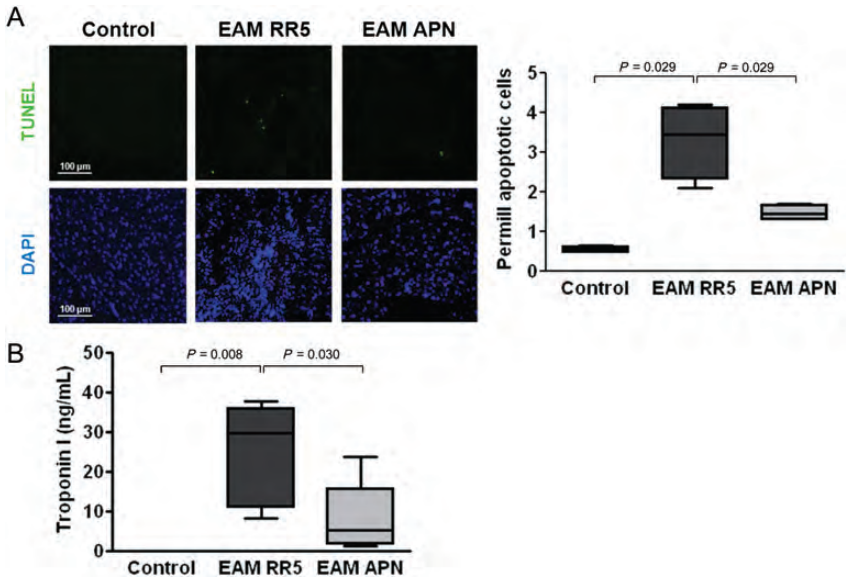

Figure 2 APN overexpression ameliorates cardiac injury in EAM. Troponin I in serum and apoptosis in hearts of mice transduced with a mouse APN expression vector (EAM APN) or control vector (EAM RR5) was determined 21 days after EAM induction. (*A*) Left: Upper panel: representative images of TUNEL stained nuclei (green). Lower panel: related images of DAPI counterstained nuclei (blue). Right: Box plot indicating the number of TUNEL positive cells relative to the total number of cells ($n = 4$ animals per group). (*B*) Troponin I serum levels are indicated ($n = 5–6$ animals per group).

APN, cells were treated with wortmannin (PI3K inhibitor), NS-398 (COX-2 inhibitor), Rp-cAMP (PKA inhibitor), and PD 098 059 (MEK1 inhibitor). Incubation of cardiomyocytes with LPS triggered a significant up-regulation of IL-6 gene expression that was almost completely abolished by APN (*Figure 3D*). Whereas NS-398 ($P = 0.001$), Rp-cAMP ($P = 0.001$), and PD 098 059 ($P = 0.001$) all partially blocked the APN effect, wortmannin did not exert any influence (*Figure 3D*). Importantly, simultaneous addition of NS-398, Rp-cAMP, and PD 098 059 to the cell culture completely abolished the inhibitory effect of APN. Therefore, inhibition of TLR4 signal transduction by APN is mediated through COX-2-, PKA-, and MEK1-dependent mechanisms. In further experiments, a possible direct interaction of APN receptors with TLR4 was investigated. For APN receptor 1 (APN-R1), an interaction with TLR4 and its co-receptor CD14 could be determined under basic culture conditions (*Figure 3E*). This interaction was enhanced by LPS incubation. APN binding to its receptor caused a dissociation of the ligand–receptor complex from TLR4/CD14 (*Figure 3E*) resulting in the inhibition of downstream signal transduction. Those data implicate APN-R1 in the stabilization of the TLR4/CD14 signalling complex.

3.6 APN inhibits TLR4-mediated activation of immune cells

TLR4 signalling is capable of activating not only cardiac, but also immune cells. Incubation of CD14+ and CD19+ cells as well as DCs with LPS significantly increased TNFα protein expression (*Figure 4A*). APN, however, significantly inhibited TNFα expression after TLR4 ligation in all three types of immune cells. In order to corroborate these findings, splenocytes from WT and APN-KO mice were isolated and cultured *in vitro*. As illustrated in *Figure 4B* and Supplementary material online, *Figure S3B*, splenocytes derived from APN-KO mice exhibited a significant increase in the mRNA expression of pro-inflammatory cytokines TNFα, IL-6 and IL-12, the chemokine CCL2 as well as ICAM-1 following TLR4 ligation compared with their WT littermates, implicating that APN deficiency promotes activation of immune cells triggered by TLR4 signalling. TLR4-mediated DC activation represents an essential trigger for EAM induction.[6] Therefore, bone marrow-derived DCs from APN-KO and WT mice were stimulated with LPS. TLR4 stimulation of DCs from APN-KO mice resulted in significantly increased expression of the activation marker CD86 when compared with their WT littermates ($P = 0.001$, *Figure 4C*) that was attenuated by APN. Moreover, DCs from APN-KO mice displayed an enhanced priming capacity following TLR4 ligation (*Figure 4D*) as they exhibited an increased ability to induce proliferation of co-cultivated T cells ($P = 0.008$). Taken together, those data in immune cells corroborate our findings in cardiac cells and demonstrate that inhibition of TLR4 signal transduction by APN is functional in both cell types. Importantly, they underline the effective role of APN in inhibiting TLR4-dependent priming and activation of immune cells that is essential for EAM induction.

3.7 APN attenuates TLR4-mediated migration of immune cells

In order to further support our hypothesis that APN inhibits TLR4 triggered cardiac inflammation and injury, the interaction between cardiac and immune cells was examined. First, migration of immune cells towards TLR4 stimulated cardiac cells was analysed. Up-regulation of CCL2 and ICAM-1 expression in response to TLR4 ligation should facilitate the migration of immune cells into the myocardium leading to increased accumulation of activated immune cells as observed following EAM induction. Indeed, TLR4 ligation on cardiomyocytes ($P = 0.008$) and fibroblasts ($P = 0.004$) by LPS significantly increased splenocyte migration (*Figure 5A*). However, incubation with APN inhibited the TLR4-mediated increase of splenocyte migration to cardiomyocytes ($P = 0.032$) and fibroblasts ($P = 0.004$), respectively. Of note, APN exhibited no detectable effect on migration of splenocytes to

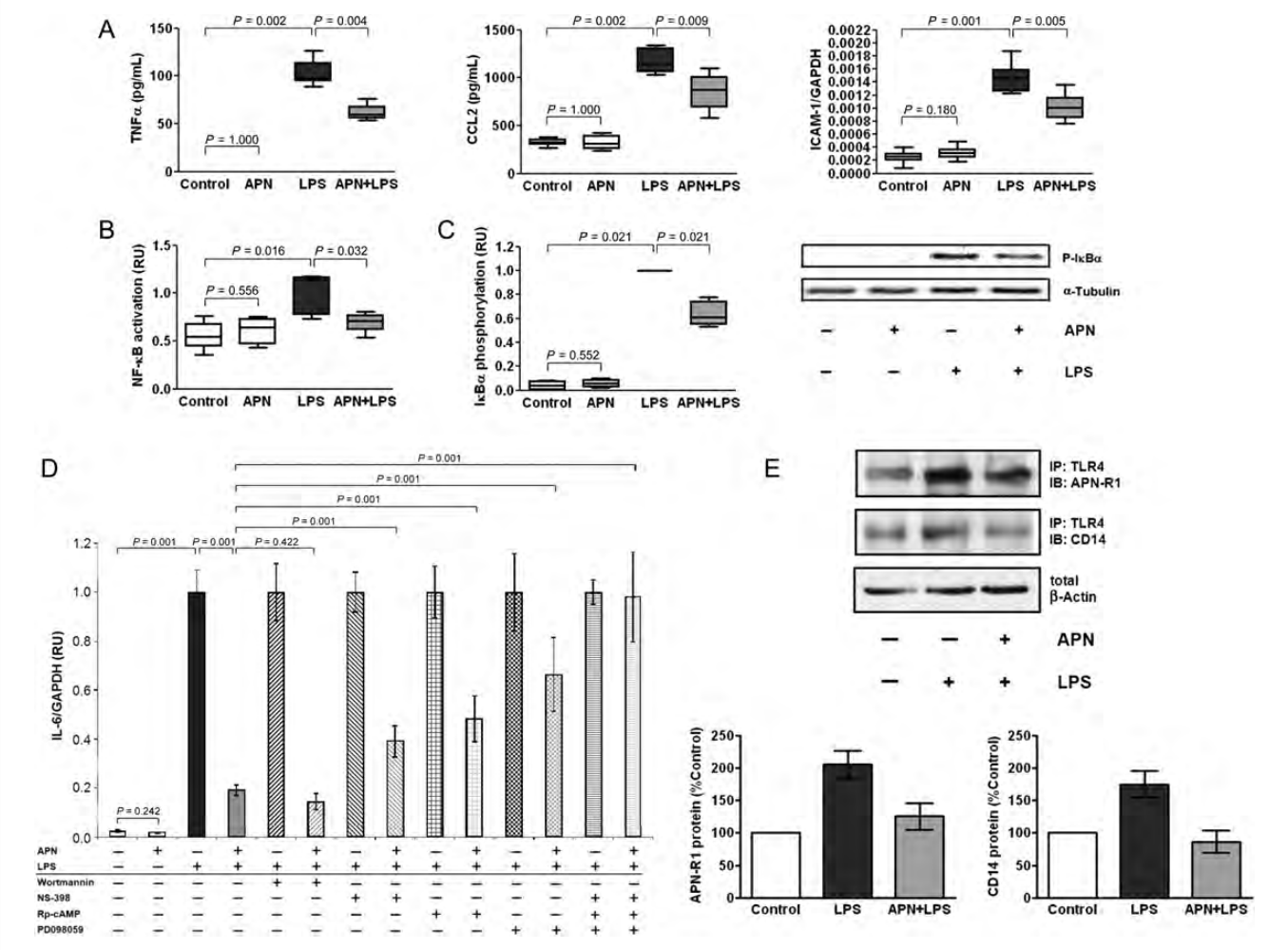

Figure 3 APN inhibits expression of a TLR4-mediated inflammatory phenotype on cardiomyocytes. (*A*) Cardiomyocytes were incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) for 18 h before stimulation with or without LPS (1 μg/mL) for 6 h. Expression of TNFα, CCL2 (ELISA), and ICAM-1 (qRT-PCR) was quantified ($n = 6$). (*B*) Cardiomyocytes were incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) before stimulation with or without LPS (1 μg/mL) for 90 min. NF-κB activation was determined by ELISA ($n = 4–5$). (*C*) Cardiomyocytes were incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) before stimulation with or without LPS (1 μg/mL) for 90 min. IκBα phosphorylation was quantified by immunoblot ($n = 4$). Box plot indicating IκBα phosphorylation normalized to α-Tubulin in relative units. (*D*) Cardiomyocytes were pre-treated with wortmannin (1 μmol/L), NS-398 (10 μmol/L), Rp-cAMP (100 μmol/L), PD 098 059 (20 μmol/L) or vehicle for 1 h, incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) for 18 h before stimulation with or without LPS (1 μg/mL) for 6 h. mRNA expression of IL-6 was determined by qRT-PCR. Results are presented as mean ± SEM in relative units ($n = 6–7$). (*E*) APN-R1 co-localizes with TLR4/CD14 signalling complex. Cardiomyocytes were incubated with LPS (1 μg/mL), APN (10 μg/mL), or vehicle (Albumin 10 μg/mL) for 3 h. Immunoprecipitation (IP) was performed as indicated and target proteins visualized by immunoblot. Bar graphs indicating mean ± SEM for respective immunoblots ($n = 3$ independent experiments per group).

unstimulated cardiac cells. Those data indicate that the inhibition of chemokine and adhesion molecule expression by APN might explain at least in part the attenuation of cardiac immune cell accumulation following APN gene transfer in EAM.

3.8 APN attenuates TLR4-mediated cardiomyocyte apoptosis

Persistent accumulation of activated immune cells in the areas of inflammation results in injury of surrounding cardiac cells.[1] Therefore, in a second interaction experiment *in vitro*, the effect of APN on TLR4-activated cardiomyocytes in co-culture with immune cells was studied. Apoptosis of cardiomyocytes co-cultivated with freshly isolated rat splenocytes was significantly increased in the presence of LPS (*Figure 5B* and *C*). Co-incubation with APN significantly attenuated the TLR4-mediated increase in cardiomyocyte apoptosis ($P = 0.008$). APN alone, however, had no detectable effect on apoptosis of cardiomyocytes. These data implicate that attenuation of the expression of an inflammatory phenotype on cardiac cells and inhibition of immune cell activation may contribute to the attenuation of cardiac injury following APN overexpression in EAM.

4. Discussion

In this study, we report for the first time APN interference with TLR4 signalling attenuating myocardial inflammation and injury in EAM. APN not only ameliorated activation of immune cells by TLR4 but inhibited

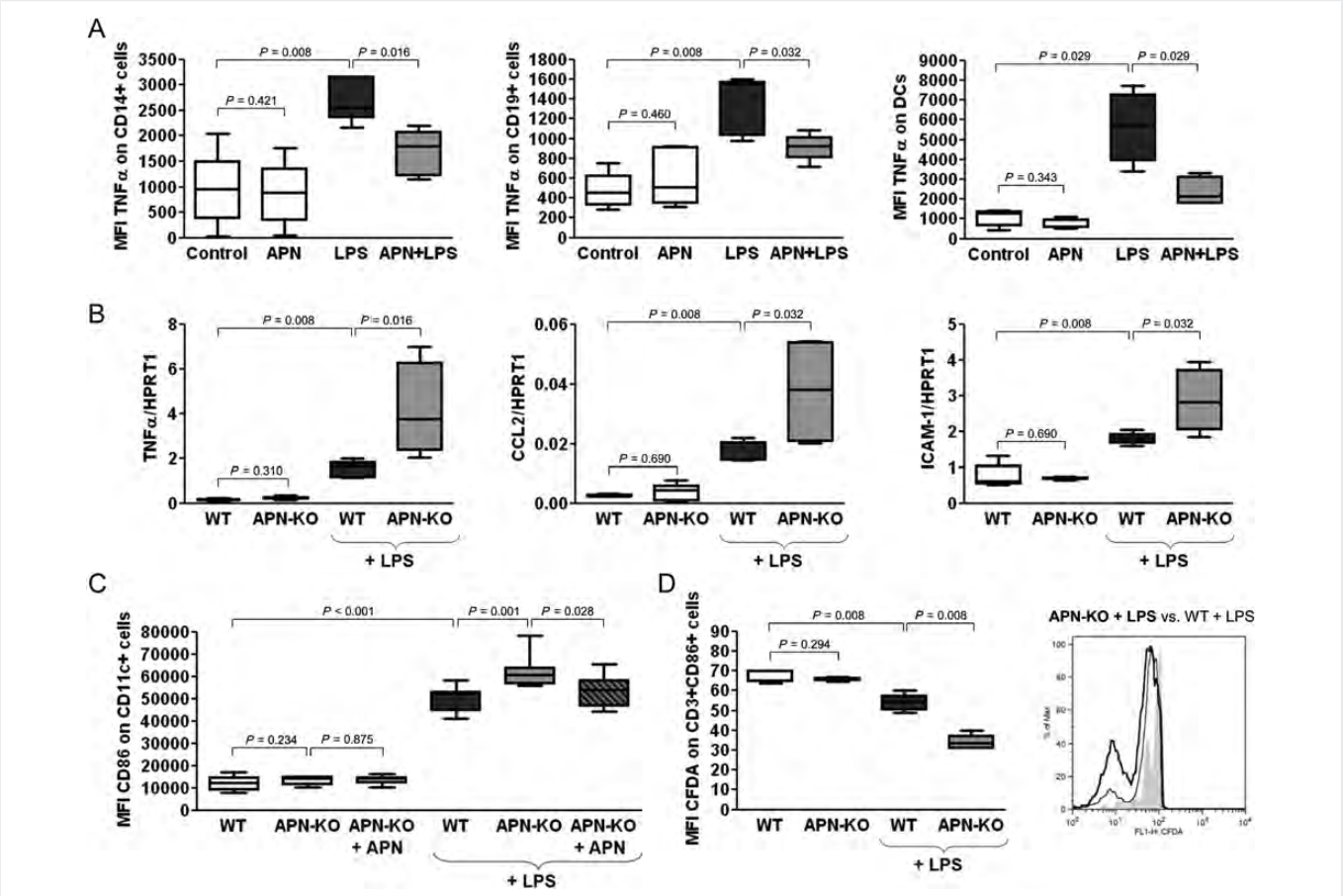

Figure 4 APN suppresses TLR4-mediated activation of immune cells. (*A*) Human CD14+ (monocytes), CD19+ (B cells), and dendritic cells (DCs) were incubated with APN (3 μg/mL) or vehicle for 24 h before stimulation with or without LPS (100 ng/mL) for 16 h. TNFα expression was determined by FACS ($n = 4$). (*B*) Splenocytes from APN-KO and WT mice were stimulated with or without LPS (100 ng/mL) for 3 h. mRNA expression of TNFα, CCL2, and ICAM-1 was determined by qRT-PCR ($n = 4–5$). (*C*) DCs from APN-KO and WT mice were stimulated with or without LPS (100 ng/mL) in the presence or absence of APN (3 μg/mL) for 24 h. Expression of CD86 on activated DCs (CD11c+ CD86+ cells) was determined by FACS. Box plot illustrates DC activation status ($n = 8$). (*D*) DCs from APN-KO and WT mice were stimulated with or without LPS (100 ng/mL) for 24 h before being added to allogenic CFDA-labelled splenocytes for 4 days. T-cell proliferation was determined by FACS ($n = 5$). Box plot illustrates DC-mediated proliferation of activated T cells (CD3+ CD86+ cells).

the expression of an inflammatory phenotype in cardiomyocytes and fibroblasts within the heart mediated by TLR4 ligation and thereby interfered with attraction and activation of immune cells by TLR4-activated cardiac cells. Mechanistically, APN diminished TLR4-dependent IκBα phosphorylation and NF-κB activation in a COX-2-, PKA-, and MEK1-dependent manner and inhibited the interaction of its receptors with the TLR4/CD14 complex (*Figure 6*).

In our *in vivo* model, cardiac TLR4 expression was down-regulated following APN gene transfer. However, attenuated cardiac infiltration with CD3+, CD14+, and CD45+ cells that express TLR4 was determined after APN overexpression. Immune cells express high quantities of TLRs. Therefore, decreased expression of TLR4 in EAM following APN gene transfer might be secondary due to reduced inflammatory cell infiltration. In line with this hypothesis, APN deficient mice showed no difference of key TLR4 signalling components and TLR4 on cardiac and immune cells was not regulated by APN even following LPS stimulation *in vitro*. However, down-regulation of TLR4 by yet unknown mechanisms in EAM *in vivo* remains a possible explanation, but early regulation of TLR4 signalling has been shown to be more important than control of TLR4 expression since low levels of TLR4 can enable signalling.[20] Our *in vitro* experiments corroborate these findings. APN inhibited TLR4-mediated phosphorylation of IκBα with subsequent translocation of NF-κB in a COX-2-, PKA-, and MEK1-dependent manner leading to diminished expression of TLR4-dependent genes such as TNFα, IL-6, IL-12, CCL2, and ICAM-1 in cardiac and immune cells *in vitro*. Moreover, APN-R1 directly interacts with the TLR4/CD14 signalling complex. After ligand binding, APN-R1 dissociates from TLR4 and CD14, thereby inhibiting downstream signalling following LPS stimulation. This interesting finding clearly needs more investigation in the future.

In line with these observations, interaction of activated cardiac cells, i.e. cardiomyocytes stimulated by TLR4 ligation with immune cells, i.e. splenocytes, was significantly inhibited by APN. Here we show that TLR4 ligation leads to the expression of an inflammatory phenotype on cardiomyocytes characterized by up-regulation of ICAM-1 and the pro-inflammatory cytokines TNFα and IL-6. Not only TNFα and IL-6, but also up-regulated CCL2 from cardiac cells induce homing and activation of immune cells. CCL2 has been shown to play a major role in regulating migration of monocytes, T cells, and natural killer (NK) cells

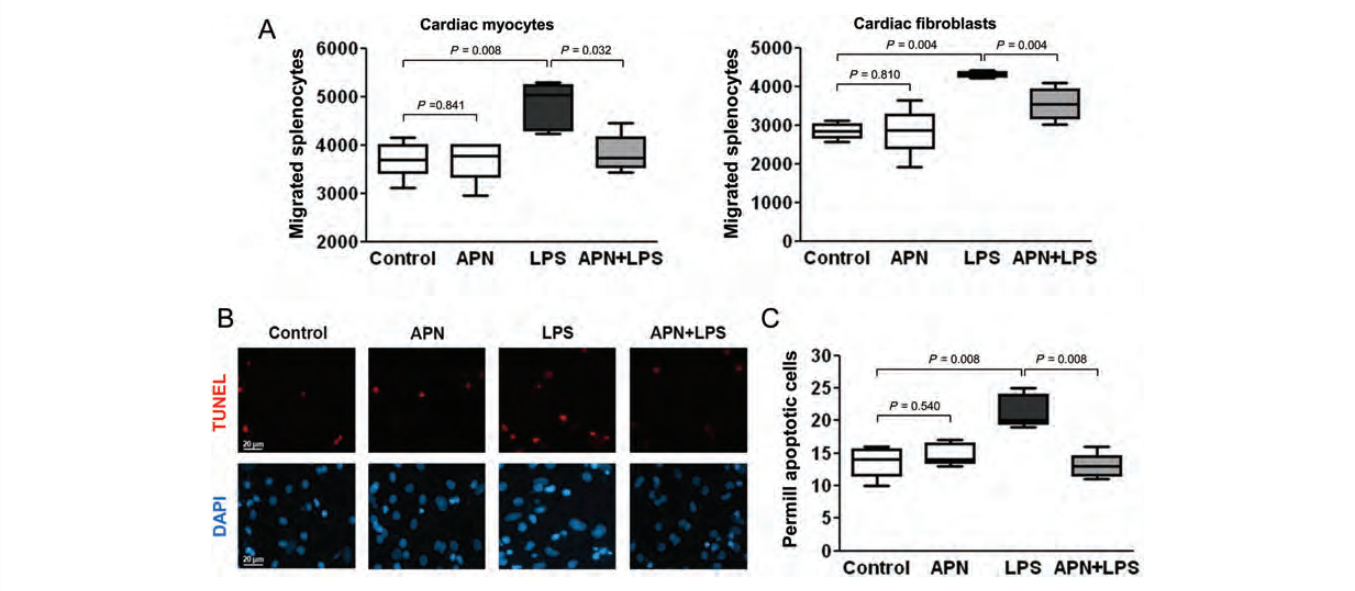

Figure 5 APN attenuates TLR4-mediated migration of splenocytes and apoptosis of cardiomyocytes after co-cultivation with splenocytes. (*A*) Cardiomyocytes and fibroblasts were incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) for 18 h before stimulation with or without LPS (1 μg/mL) for 24 h. Migration of co-cultivated CFDA-labelled splenocytes towards cardiac cells was quantified by FACS ($n = 5$). (*B*) Cardiomyocytes were incubated with APN (10 μg/mL) or vehicle (Albumin 10 μg/mL) for 18 h before co-cultivation with splenocytes in the presence or absence of LPS (1 μg/mL) for 24 h. Apoptosis of cardiomyocytes was quantified by TUNEL staining after removal of splenocytes. Upper panel: representative images of TUNEL stained nuclei (red). Lower panel: related images of DAPI counterstained nuclei (blue). (*C*) Box plot indicating the number of TUNEL positive cells relative to the total number of cells ($n = 5$).

to an inflammatory focus. Recently, it has been shown that CCL2 is up-regulated in EAM in rodents as well as patients with myocarditis, and blocking of CCL2 with monoclonal antibodies reduced the severity of autoimmune myocarditis.[21] In contrast, CCL2 overexpression within the heart leads to the induction of myocarditis.[22] Moreover, mice deficient in CCR2, the receptor of CCL2, exhibit a reduced prevalence and severity of EAM.[22] In our model, EAM led to a pronounced up-regulation of cardiac CCL2 that was significantly attenuated following APN gene-transfer. Furthermore, APN significantly diminished CCL2 expression following TLR4 ligation in cardiac cells *in vitro*. Indeed, we show a diminished migration of splenocytes to TLR4- activated cardiomyocytes and fibroblasts *in vitro*. Moreover, less CD3+, CD14+, and CD45+ immune cells were detected in the myocardium of EAM mice 21 days following APN gene transfer, indicating that APN interferes with TLR4-mediated up-regulation of CCL2 *in vivo*.

Besides CCL2, expression of the TLR4 target genes TNFα, IL-6, IL-12 was diminished in EAM following APN gene transfer. TNFα is a major pro-inflammatory cytokine inducing apoptosis, ROS, and reduction of left-ventricular ejection fraction.[23] Transgenic mice cardio-specifically overexpressing TNFα develop cardiomyopathy characterized by extensive cardiac inflammation,[23] and increased plasma concentrations of TNFα are found in patients with congestive heart failure and dilated cardiomyopathy.[13] IL-6 is essential in the pathogenesis of EAM, because its deletion leads to diminished prevalence and severity of autoimmune myocarditis due to a lack of expansion of critical CD4+ T cells as well as diminished production of complement C3, a crucial factor for the development of myocarditis.[14] Moreover, IL-6 mediates the differentiation of T helper 17 (Th17) cells, that play an important role in the initiation of EAM. Similar to IL-6, IL-12 promotes the development of autoimmune myocarditis by regulating autoreactive CD4+ T cell proliferation as well as autoreactive CD8+ T cell differentiation.[24] Taken together, down-regulation of TNFα, IL-6, and IL-12 expression levels by APN in a TLR4-dependent manner might in part explain the observed attenuation of cardiac inflammation in EAM following APN overexpression.

Activated immune cells play an important role in cardiovascular inflammation by removing cell debris and pathogens, but chronic inflammation such as in EAM leads to tissue injury.[1] In fact, chemotaxis and activation of splenocytes induced by TLR4-mediated up-regulation of CCL2 and TNFα resulted in increased apoptotic cell death *in vitro* that was inhibited by APN in our study. Furthermore, APN gene transfer in EAM mice was associated with attenuation of myocardial apoptosis. Tissue injury leads to release of extracellular matrix components such as hyaluronan and fibronectin extra domain A (FDA), plasma proteins (fibrinogen), and cytoplasmatic proteins (HSP60) that are able to activate TLR4 signalling and to induce apoptotic cell death.[2,25] The importance of TLR4 activation on cardiac and immune cells has been shown in several injury models. In ischaemia–reperfusion injury, myocardial and not immune cell TLR4 is the primary mediator for cardiac depression as has been shown for sepsis-related cardiac dysfunction.[26] In line with our observations, Ao *et al.*[27] demonstrated that myocardial tissue TLR4 rather than neutrophil TLR4 is the determinant of neutrophil infiltration following I/R, implicating TLR4 in the homing of leucocytes following tissue injury and inflammation.

Other mechanisms having been shown to be involved in cardioprotection in EAM are suppression of TLR4-dependent DC activation and priming as well as inhibition of differentiation of T cells.[6] Timely activation of TLR4 (innate immunity) together with CD40 (adaptive immunity) on DCs is essential for the induction of EAM.[6] Therefore, APN-induced suppression of TLR4 signalling might interfere with differentiation, activation, and antigen presentation by DCs. In line with this hypothesis, mice deficient in the downstream adaptor molecule MyD88 are protected from EAM since MyD88 signalling in DCs is essential to prime

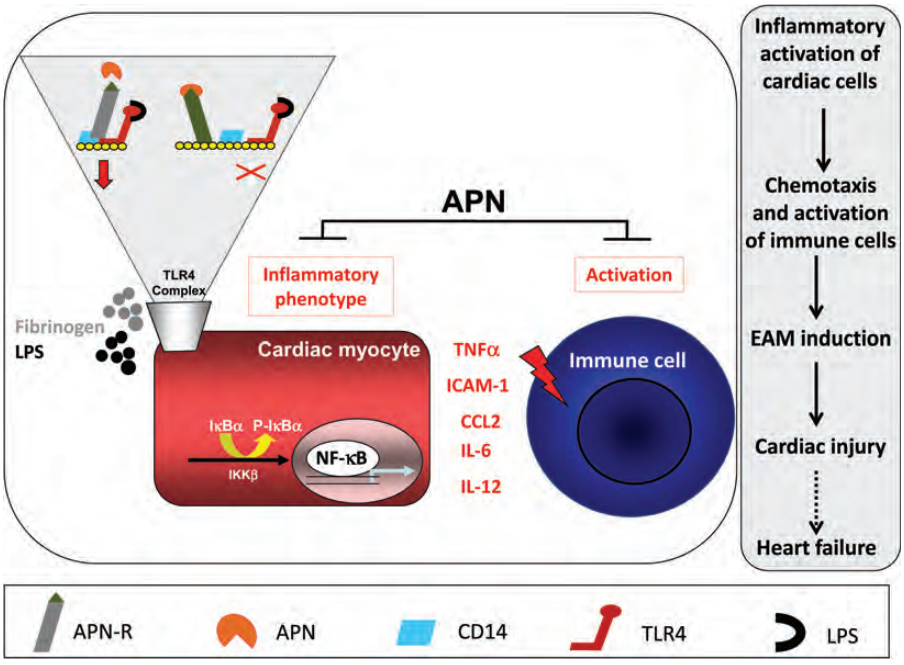

Figure 6 APN protects against inflammation and injury in EAM by interfering with TLR4 signalling in cardiac and immune cells. Binding of TLR4 by exogenous (LPS) or endogenous ligands (HSPs, hyaluronan, fibrinogen) on cardiac and immune cells leads to NF-κB-mediated expression of a pro-inflammatory phenotype. TLR4-activated cardiomyocytes and fibroblasts up regulate cytokines, chemokines, and adhesion molecules that induce chemotaxis and activation of immune cells. Activated immune cells release important pro-inflammatory cytokines mandatory for the induction and progression of EAM. Persistent cardiac accumulation of activated immune cells induces tissue injury. APN protects against inflammation and injury in autoimmune myocarditis by binding to APN-R1, thereby destabilizing the TLR4/CD14 signalling complex and inhibiting downstream signalling.

heart specific CD4+ T cells[28] in a TNFα-dependent manner. TNFα has an important role in the induction of EAM and was significantly down-regulated following APN gene transfer in our study. *In vitro* blocking of TNFα by a specific antibody inhibits antigen-specific DC priming and proliferation of CD4+ T cells and recombinant TNFα restores proliferative responses of CD4+ T cells in MyD88 deficient DCs, indicating that MyD88-regulated TNFα is important for CD4+ T cell-dependent EAM in our model.[28] Therefore, down-regulation of TNFα by APN might inhibit EAM by attenuating priming of CD4+ T cells. In this regard, APN inhibited the up-regulation of TNFα in DCs, the activation of DCs and the DC-mediated priming of antigen-specific T cells following TLR4 ligation in our study. Moreover, TLR4-deficient mice develop markedly reduced myocarditis after infection with enterovirus.[29] Further, we have recently shown APN to inhibit the expansion of antigen-specific T cells by attenuation of proliferation and induction of apoptosis.[10] Although this process is not TLR4-dependent, APN-mediated diminished proliferation of myosin-specific T cells might attenuate tissue injury in our CD4+ T cell-dependent EAM model.

One limitation of the study is not being able to use APN deficient mice in our *in vivo* model because of a different background (C57BL/6). However, a life-time increase or absence of APN may induce changes in multiple systems that may obscure direct modulatory effects of APN on inflammation *in vivo*. Specific questions that arose from our EAM *in vivo* studies could be confirmed in APN-deficient animals. Therefore, confirming certain aspects, i.e. inhibition of TLR4 signalling by APN in APN-KO mice together with results gathered in rat neonatal cardiomyocytes and fibroblasts as well as human immune cells strengthen the data obtained in BALB/c mice by providing evidence for a general applicability of APN effects on TLR signalling.

In conclusion, our data implicate at least two different mechanisms for protection resulting from APN overexpression in EAM. First, inhibition of EAM induction by suppression of TLR4-dependent DC activation resulting in attenuated initial priming of autoreactive CD4+ T cells. Secondly, inhibition of EAM progression by down-regulation of TLR4-dependent pro-inflammatory gene expression in cardiac and immune cells resulting in attenuated activation and interaction of both cell types limiting myocardial injury.

Beyond EAM, our findings have wider implications and may account for a multitude of anti-inflammatory effects described for APN since endogenous ligands of TLR4 are released in multiple types of cardiovascular injury. The described APN-mediated inhibition of TLR4 signalling might be a general anti-inflammatory mechanism confining inflammation in cardiovascular diseases among others in atherosclerosis,[30] cardiac hypertrophy/left-ventricular remodelling,[3] sepsis, and inflammatory cardiomyopathy.[13]

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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